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γ-*Tubulin37C* and γ-*tubulin ring complex protein 75* Are Essential for *bicoid* RNA Localization during *Drosophila* Oogenesis

Frank Schnorrer,[1] Stefan Luschnig,[2] Iris Koch, and Christiane Nüsslein-Volhard
Max-Planck-Institut für Entwicklungsbiologie
Abteilung Genetik
Spemannstr. 35
72076 Tübingen
Germany

Summary

***bicoid* (*bcd*) RNA localization requires the activity of *exuperantia* and *swallow* at sequential steps of oogenesis and is microtubule dependent. In a genetic screen, we identified two novel genes essential for *bcd* RNA localization. They encode maternal γ-*Tubulin37C* (γ*Tub37C*) and γ-*tubulin ring complex protein 75* (*Dgrip75*), both of which are γ-tubulin ring complex components. Mutations in these genes specifically affect *bcd* RNA localization, whereas other microtubule-dependent processes during oogenesis are not impaired. This provides direct evidence that a subset of microtubules organized by the γ-tubulin ring complex is essential for localization of *bcd* RNA. At stage 10b, we find γTub37C and Dgrip75 anteriorly concentrated and propose the formation of a microtubule-organizing center at the anterior pole of the oocyte.**

Introduction

Asymmetry along the anterior-posterior axis of early *Drosophila* embryos originates from localized mRNAs at opposite poles of the freshly laid egg (Riechmann and Ephrussi, 2001). *bicoid* (*bcd*) and *nanos* (*nos*) mRNAs are concentrated at the anterior and posterior poles, respectively (Berleth et al., 1988; Wang and Lehmann, 1991). Bcd and Nos proteins spread from their localized sources and generate the primary morphogen gradients patterning the anterior-posterior axis of the *Drosophila* embryo (Driever and Nüsslein-Volhard, 1988; Wang and Lehmann, 1991).

Previous genetic screens have identified *exuperantia* (*exu*) and *swallow* (*swa*) as essential factors for *bcd* mRNA localization (Frohnhöfer and Nüsslein-Volhard, 1987). The process of *bcd* RNA localization is initiated early during oogenesis, when *bcd* RNA is produced in the nurse cells and assembled into large particles in the cytoplasm (Berleth et al., 1988; St Johnston et al., 1989). Exu protein is concentrated in electron-dense structures, termed sponge bodies, in the nurse cell cytoplasm, in which the assembly of Exu- and *bcd* RNA-containing particles is likely to occur (Wilsch-Bräuninger et al., 1997). These particles move into the oocyte in a microtubule-dependent manner, and *bcd* RNA localizes, initially together with Exu protein, at the anterior cortex of the oocyte (Cha et al., 2001; Theurkauf and Hazelrigg, 1998; Wang and Hazelrigg, 1994). This anterior localization of *bcd* RNA is disrupted in *exu* mutants (Berleth et al., 1988; St Johnston et al., 1989).

While Exu appears to be dispensable as soon as *bcd* RNA reaches the oocyte (Cha et al., 2001), *swallow* (*swa*) is required for *bcd* RNA localization within the oocyte. In *swa* mutants the initial localization is normal, but *bcd* RNA fails to stay anteriorly from stage 10b of oogenesis onward (St Johnston et al., 1989; Stephenson et al., 1988). In a wild-type oocyte, Swa protein colocalizes with *bcd* RNA at the anterior cortex at stage 10b in a microtubule-dependent manner. Swa interacts with dynein light chain and might be transported to the microtubule minus ends at the anterior pole by the dynein motor complex. Swa localization is independent of *exu* and *bcd* RNA, demonstrating that this localization does not require the early *bcd* RNA localization machinery acting in the nurse cells (Schnorrer et al., 2000).

How the localization of *bcd* RNA is achieved in the oocyte is not well understood. Depolymerization of microtubules abolishes the anterior *bcd* RNA concentration at stage 9 and also at stage 10 of oogenesis (Pokrywka and Stephenson, 1991, 1995), indicating a continuous requirement for the microtubule skeleton in order to localize and maintain *bcd* RNA at the anterior pole. However, drug treatment affects all microtubules at the same time, and, hence, these experiments do not allow us to distinguish different steps of the localization process or to understand its regulation. Microtubules are found in an anterior to posterior gradient at stage 9 (Theurkauf et al., 1992). The current model of microtubule polarity is mainly based on the observation that a kinesin heavy chain β-galactosidase (Kin:β-gal) and an unconventional kinesin β-galactosidase fusion (Nod:β-gal) are concentrated at the posterior and anterior pole of a stage 9 oocyte, respectively (Clark et al., 1994, 1997). Therefore, the stable microtubule minus ends are thought to localize at the anterior, whereas plus ends seem to spread to the posterior. However, this model is complicated by the finding that endogenous dynein heavy chain, a motor protein transporting cargo to the minus ends, is also concentrated at the posterior pole of stage 9 oocytes (Li et al., 1994). At later stages of oogenesis, at which *swa* has an essential function for *bcd* RNA localization, microtubules are mainly assembled subcortically, with an anterior concentration (Theurkauf et al., 1992). Their polarity and dynamics are not well described. Importantly, both Swa and *bcd* RNA localize to both poles in *gurken* (*grk*) mutant oocytes, which contain a microtubule cytoskeleton with duplicated polarity, resulting in the presumptive minus ends at the anterior and posterior poles at stage 9 (Gonzalez-Reyes et al., 1995; Schnorrer et al., 2000). The ectopic *bcd* RNA localization at the posterior of *grk* oocytes argues for an active transport of *bcd* RNA in order to reach the posterior pole and suggests that the observed anterior localization of *bcd* RNA in wild-type oocytes is not established by simply trapping the imported RNA. However, there is no genetic evidence for a connection

[1]Correspondence: frank.schnorrer@tuebingen.mpg.de
[2]Present address: Stanford University, School of Medicine, Stanford, California 94305.

between *bcd* RNA localization and the microtubule cytoskeleton.

During *Drosophila* oogenesis many different processes depend on the integrity of the microtubule cytoskeleton. It is necessary for oocyte specification in the germarium (Theurkauf et al., 1993), for movement of the oocyte nucleus from posterior pole to the future dorsal-anterior corner, and for both *bcd* and *osk* RNA transport to the anterior and posterior pole, respectively (Pokrywka and Stephenson, 1995; Theurkauf et al., 1992). To fulfill these multiple functions properly, the cytoskeleton must be tightly controlled in a temporal and spatial manner. However, there is no centrosome or apparent microtubule-organizing center (MTOC) known in the oocyte at midoogenesis, when transport of the different RNAs to the anterior and posterior pole occurs. But, under normal cellular environments, free microtubules are extremely unstable (Kirschner and Mitchison, 1986). Microtubule organization has been studied in more detail during early *Drosophila* embryogenesis. There, the γ-tubulin ring complex (γTuRC), which is composed of γ-Tubulin37C and several different γ-tubulin ring complex proteins, is located at the centrosomes, specifically at the base of a microtubule. Its ring-shaped structure serves as a template for a microtubule and allows the controlled polymerization of tubulin dimers (Moritz et al., 2000; Oegema et al., 1999). Furthermore, it prevents microtubule shrinkage at the minus ends (Wiese and Zheng, 2000).

In a genetic mosaic screen, using an in situ hybridization assay, we isolated mutations in two novel genes required for *bcd* RNA localization. These genes code for γ-Tubulin37C (γTub37C) and γ-tubulin ring complex protein 75 (Dgrip75), which are both components of the γ-tubulin ring complex. The anterior concentration of *bcd* RNA is lost after stage 10b in both mutants, thus providing direct genetic evidence that an intact microtubule skeleton is indeed essential for correct *bcd* RNA localization. We propose that the γ-tubulin ring complex establishes an MTOC at the anterior pole of the oocyte at stage 10b, which is essential to maintain *bcd* RNA localization. *γTub37C* and *Dgrip75* mutants specifically affect the function of this MTOC, whereas other microtubule-dependent processes during oogenesis remain intact.

Results

Identification of Two Novel Loci Essential for *bcd* RNA Localization

Components of the cytoskeletal machinery, which localizes *bcd* RNA to the anterior pole of the oocyte, might have multiple functions during development. Therefore, we chose the following approach in order to identify novel factors essential for *bcd* RNA localization. As part of a large F1 genetic screen for EMS-induced maternal effect mutations described in detail elsewhere (S.L. and C.N.-V., in preparation), we isolated mutant females whose germ line clone-derived eggs arrest early in development, as judged by the absence of cuticular structures (see Experimental Procedures). We inspected the ovaries of those females by situ hybridization using a *bcd* RNA antisense probe and isolated three mutations, *139-14*, *175-14*, and *224-26*, displaying an abnormal *bcd* RNA localization with 100% penetrance. *175-14* and *224-26* are two alleles of the same gene, while *139-14* represents a single allele.

139-14 and *175-14* Encode Components of the γ-Tubulin Ring Complex

Using a set of overlapping chromosomal deficiencies, we mapped *139-14* to cytological region 37C. Database searches revealed γ-*Tubulin37C* (*γTub37C*) as a candidate gene for the *139-14* mutation. *139-14* indeed failed to complement *γTub37C* alleles $\gamma Tub37C^{1}$ and $\gamma Tub37C^{3}$ and showed no detectable γTub37C protein on a Western blot with an antibody specific for the C terminus of γTub37C (Figure 1A). Therefore, we conclude that *139-14* bears a mutation in the *γTub37C* locus. *γTub37C* mutants are viable, but females are sterile. The eggs produced by mutant mothers show an arrest of nuclear divisions during early embryogenesis because microtubule polymerization and, as a consequence, spindle formation are blocked (Llamazares et al., 1999; Tavosanis et al., 1997).

Mutations in the second gene, *175-14* and *224-26*, are also viable. We mapped the mutations to region 31D-F, where *Dgrip75* (γ-tubulin ring complex protein of 75 kDa) is annotated on the basis of the sequence homology to human *Grip76*. The human homolog is part of the γ-tubulin ring complex and is located at the centrosomes of eukaryotic cells, but no mutants are described (Fava et al., 1999). We sequenced the *Dgrip75* gene from *175-14* and *224-26* flies and identified molecular lesions in both mutants. The *Dgrip75* gene of *175-14* has a nonsense mutation at base pair 911 of its mRNA, resulting in a truncation of the predicted protein after amino acid 290. *224-26* contains a missense mutation at the splice acceptor site of the 6th intron, resulting in an mRNA with a 1 bp insertion after base pair 1580, corresponding to amino acid 513 (Figure 1B). We used two internal fragments of Dgrip75 to generate a Dgrip75 polyclonal antibody. The antiserum recognizes a band of about 75 kDa that is missing in both *Dgrip75* alleles, indicating that both *Dgrip75* alleles are protein null alleles (Figure 1A). We expressed Dgrip75 fused to GFP using a maternal α-*tubulin* GAL4 line in a *175-14* background. This Dgrip75GFP fusion protein rescues the female sterility of *175-14* and its *bcd* RNA mislocalization defect (data not shown). In conclusion, *175-14* and *224-26* are mutations in the *Dgrip75* locus and will be referred to as $Dgrip75^{175}$ and $Dgrip75^{224}$, respectively.

swa, *γTub37C*, and *Dgrip75* Affect *bcd* RNA Localization at Midoogenesis

To identify the time point at which the *bcd* RNA localization pattern is disrupted in *γTub37C* and *Dgrip75* mutants, we analyzed *bcd* RNA distribution at different stages of oogenesis in comparison with wild-type and *swa* and *exu* mutants. The ring-shaped localization of *bcd* RNA at the anterior cortex of stage 8–10a oocytes forms normally in *γTub37C*, *Dgrip75*, and *swa* mutants, while it is strongly reduced in *exu* egg chambers (Figure 2A; data not shown). In stage 10b wild-type oocytes, the *bcd* RNA localization changes from the ring-shaped into a disc-shaped pattern, resulting in highest *bcd* RNA

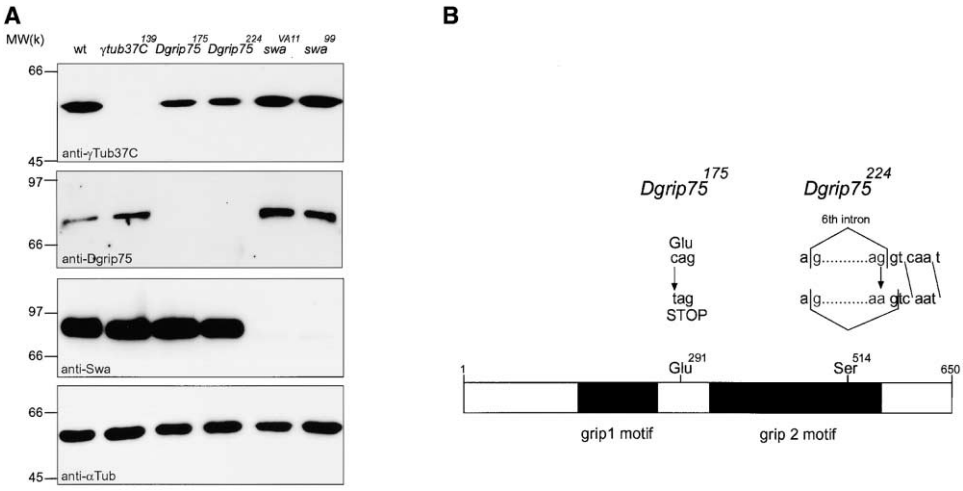

Figure 1. Characterization of γTub37C and Dgrip75 Mutant Proteins

(A) Wild-type or mutant ovary protein extracts were blotted and probed with an anti-γTub37C-specific antibody (upper panel), anti-Dgrip75 antibody (second panel), anti-Swa antibody (third panel), and anti-αTub antibody (lower panel). *γTub37C*^{139} does not contain detectable γTub37C protein. *Dgrip75*^{175} and *Dgrip75*^{224} are Dgrip75 protein null alleles.

(B) Molecular architecture of Dgrip75 protein and its alleles. Motifs conserved within the grip family are indicated according to Gunawardane et al. (2000). *Dgrip75*^{175} has a C to T change resulting in a conversion of Glu^{291} to a STOP; *Dgrip75*^{224} contains a G to A change of a splice acceptor site leading to a frame shift at Ser^{514}.

concentration at the middle of the anterior pole (Figure 2B). This transition is never observed in *swa* mutants. Instead, *bcd* RNA diffuses away from the anterior pole into the oocyte and is often seen in aggregates associated with the oocyte cortex (Figures 2B–2D; St Johnston et al., 1989). In *γTub37C* and *Dgrip75* mutants, this *swa*-dependent transition occurs at least partially, since *bcd* RNA is found at the center of the anterior pole at stage 10b, although less in *γTub37C* compared with wild-type (Figure 2B). Slightly later, *bcd* RNA spreads into the

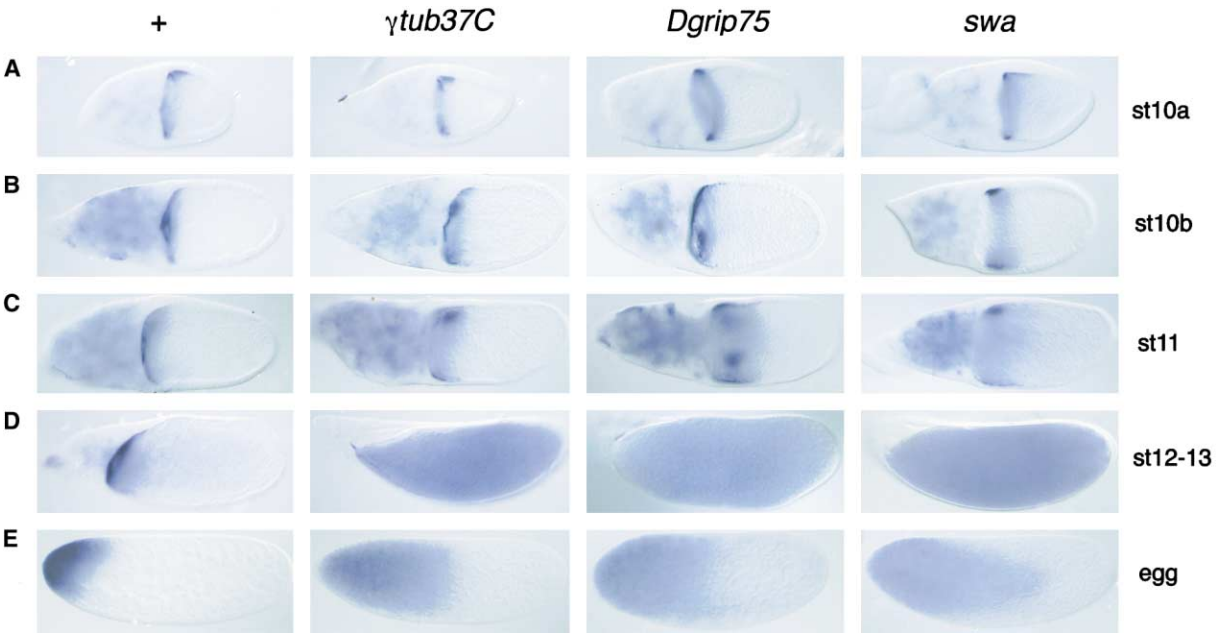

Figure 2. *bcd* RNA Distribution in Wild-type and in *γTub37C*, *Dgrip75*, and *swa* Mutants at Different Stages of Oogenesis and in Early Eggs

Wild-type, left row; *γTub37C*, middle left row; *Dgrip75*, middle right row; *swa*, right row.

(A) Stage 10a.

(B) Stage 10b.

(C) Stage 11.

(D) Stage 12 to 13; see text for description.

(E) Zero to 1 hr old eggs. The observed asymmetry of *bcd* RNA in *γTub37C*, *Dgrip75* and *swa* mutant eggs is due to the *bcd* RNA destabilization activity of the posterior system (see Berleth et al., 1988).

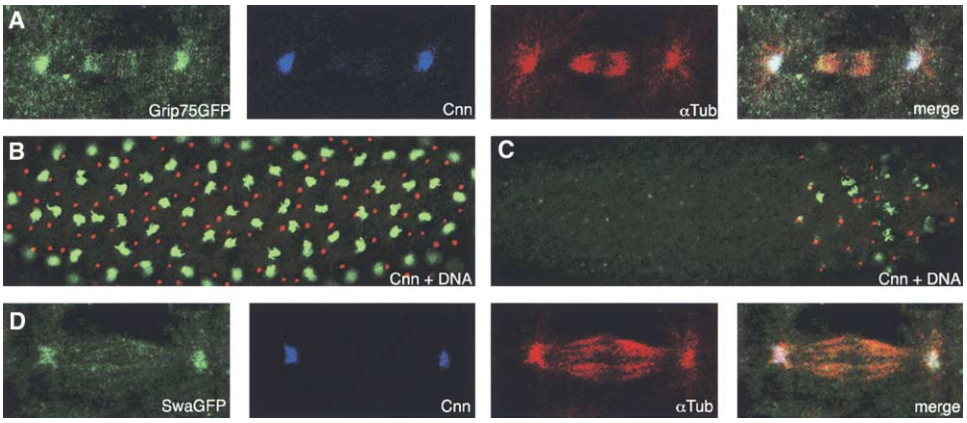

Figure 3. Dgrip75GFP and SwaGFP Distribution in Early Embryos and Characterization of a *swa* Nuclear Clevage Phenotype

Dgrip75GFP and SwaGFP localize to centrosomes of wild-type embryos at syncytial bastoderm stages (A and D). Irregular centrosome distribution in *swa* embryos (B and C).

(A) High-power confocal image of an anaphase spindle showing Dgrip75GFP in green, Centrosomin in blue, and α-tubulin in red. Right panel shows the three-color overlay. Dgrip75GFP localization is not as restricted to the centrosome as Cnn.

(B and C) Confocal surface view of a wild-type (B) and a *swa* protein null embryo raised at 18°C (C). Centrosomes are shown in red by Centrosomin staining, DNA is shown in green by oligreen. Centrosomes and nuclei have a regular pattern in wild-type (B); this pattern is disrupted in *swa* null mutants (C). *swa* embryos often contain large regions depleted of centrosomes and nuclei; sometimes centrosomes divide and accumulate without nuclei.

(D) High-power confocal image of a metaphase spindle showing SwaGFP in green, Centrosomin in blue, and α-tubulin in red. Right panel shows the three-color overlay. SwaGFP is concentrated at, but not restricted to, the centrosomes.

oocyte, forming aggregates similar to those seen in *swa* mutants (Figure 2C), leading to a uniform distribution of *bcd* RNA at late stages of oogenesis (Figure 2D). From these data we deduce that γ*Tub37C*, *Dgrip75*, and *swa* act in sequential steps of the *bcd* RNA localization process. *swa* is essential for the "ring to disc" transition of the *bcd* RNA pattern, which coincides with the anterior concentration of Swa protein at stage 10b, whereas γ*Tub37C* and *Dgrip75* are required to maintain *bcd* RNA at the anterior pole. After stage 10b the *bcd* RNA mislocalization pattern is indistinguishable in all three mutants (see Figure 2D).

Dgrip75 Is a Component of Centrosomes in Early Embryos and Colocalizes with SwaGFP

γTub37C and Dgrip75 are both components of the microtubule cytoskeleton. γTub37C is known to localize at centrosomes of early *Drosophila* embryos and is essential for organization and control of microtubule growth (Gunawardane et al., 2000; Tavosanis et al., 1997). Before analyzing the molecular function of γ*Tub37C* and *Dgrip75* during oogenesis, we looked at embryos laid from *Dgrip75* mutant mothers and, as in γ*Tub37C* embryos, we could not detect any spindles or dividing nuclei (data not shown). Therefore, we conclude that Dgrip75 is an essential component of the γ-tubulin ring complex, since *Dgrip75* mutants display a complete loss of spindle function indistinguishable from γ*Tub37C* mutants.

A maternally expressed Dgrip75GFP fusion rescues the spindle defect of *Dgrip75* mutants, and nuclear divisions occur normally. Dgrip75GFP is concentrated at centrosomes of early embryos and colocalizes with γTub37C and Centrosomin (Cnn) throughout the cell cycle (Figure 3A; see Supplemental Movie S1 at http://www.developmentalcell.com/cgi/content/full/3/5/685/DC1; data not shown). These rescue data indicate that the Dgrip75GFP fusion protein is functional and, hence, that it is likely to recapitulate the endogenous Dgrip75 distribution.

Interestingly, *swa* null mutants also show a phenotype during embryonic nuclear cleavage stages (Frohnhöfer and Nüsslein-Volhard, 1987; Hegde and Stephenson, 1993). Nuclear divisions are often abnormal from the first divisions onward: large regions in the embryo are depleted of nuclei, whereas sometimes centrosomes divide without nuclei (compare Figure 3B with 3C). Probably as a consequence of the irregular distribution of centrosomes during the early divisions, centrosomes are not stably anchored when they arrive at the cell cortex but move backward and forward (see Supplemental Movie S2 at http://www.developmentalcell.com/cgi/content/full/3/5/685/DC1). The centrosomes that form a spindle in *swa* embryos contain normal amounts of γTub37C and Cnn (data not shown). Endogenous Swa protein is targeted for degradation shortly after egg deposition and is undetectable after one hour of development (Schnorrer et al., 2000). However, a maternally expressed SwaGFP fusion perdures longer and allows the analysis of its distribution during nuclear cleavages. Strikingly, we find it concentrated together with γTub37C and Cnn at the centrosomes of the early embryo (Figure 3D), suggesting that Swa may function together with γTub37C and Dgrip75 to organize and coordinate the nuclear spindles of the first divisions. Like Dgrip75GFP and γTub37C, SwaGFP is not exclusively found at the centrosomes but is also distributed on the microtubules of the mitotic figure (see Figure 3). Time-lapse analysis of the SwaGFP distribution shows that the SwaGFP concentration at centrosomes declines after mitosis and is reestablished within the next interphase (see Supplemental Movie S3 at http://www.developmentalcell.com/cgi/content/full/3/5/685/DC1).

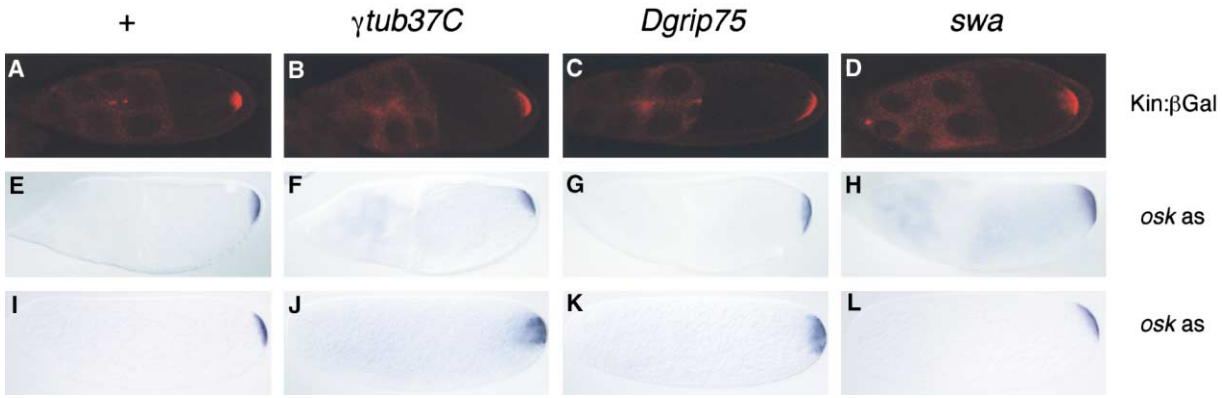

Figure 4. Posterior Transport Is Unaffected in γ*Tub37C*, *Dgrip75*, and *swa* Mutant Oocytes

Kin:β-gal (A–D) and *osk* RNA (E–L) distribution in wild-type (A, E, and I) and in γ*Tub37C* (B, F, and J), *Dgrip75* (C, G, and K), and *swa* mutants (D, H, and L).

(A–D) Kin:β-gal at stage 9.

(E–H) *osk* RNA at stage 10b.

(I–L) *osk* RNA in 0–1 hr eggs. The tight association of *osk* RNA with the posterior cortex is lost in γ*Tub37C* and *Dgrip75* mutant eggs.

Microtubule Network in γ*Tub37C*, *Dgrip75*, and *swa* Mutant Oocytes

Since γ*Tub37C*, *Dgrip75*, and *swa* mutants display spindle defects during embryogenesis, we asked whether the microtubule network is also altered during oogenesis. To investigate function and polarity of the microtubules in γ*Tub37C* and *Dgrip75* ovaries, we first analyzed transport to the posterior pole of the oocyte. We looked for Kin:β-gal and *osk* RNA, whose posterior concentration depends on endogenous *kinesin heavy chain* function (Brendza et al., 2000). Kin:β-gal and *osk* RNA localization are normal in γ*Tub37C* and *Dgrip75* oocytes (Figures 4A–H), and *osk* RNA remains concentrated at the posterior pole of early γ*Tub37C* and *Dgrip75* embryos (Figures 4I–4L). However, the posterior association of *osk* RNA with the cortex is not as tight as in wild-type or *swa* embryos. This suggests that transport to the posterior pole is functional in γ*Tub37C* and *Dgrip75* mutants, but that a late anchoring step of *osk* RNA is impaired.

Next, we analyzed transport to the anterior pole. Previously, we showed that Swa localization at the anterior pole of stage 10 oocytes requires intact microtubules (Schnorrer et al., 2000). Hence, we used Swa to investigate the function of microtubule-dependent transport and found that it is localized normally at the anterior pole of γ*Tub37C* and *Dgrip75* oocytes from stage 10b onward (Figures 5A–5F), indicating that γ*Tub37C* and *Dgrip75* are not required for the anterior localization of Swa. For a further characterization of the microtubule network, we used the microtubule minus end marker Nod:β-gal (Clark et al., 1997). Interestingly, we observe a change of the Nod:β-gal localization from a ring-shaped pattern at stage 9 into a disc-shaped pattern at stage 10b, which is reminiscent of the change in the *bcd* RNA localization pattern (Figures 5G and 5J). Nod:β-gal colocalizes with Swa and *bcd* RNA at stage 10b of wild-type and *swa* mutant oocytes (Figures 5J and 5L). This suggests that *swa* is not required for Nod:β-gal transport to the anterior pole, indicating that the microtubule cytoskeleton is not generally disrupted in *swa* mutants. However, in γ*Tub37C* mutant oocytes, the anterior localization of Nod:β-gal is reduced at stage 9, and it is not detectable anymore at the anterior pole at stage 10b (Figures 5H and 5K), indicating that transport to the microtubule minus ends is not functioning properly in these oocytes. Furthermore, these data imply a continuous requirement of minus end-directed transport in order to maintain the anterior localization of Nod:β-gal and of *bcd* RNA.

In order to address the question of whether γ*Tub37C* and *Dgrip75* mutants specifically affect a subset of microtubule function at the time when *bcd* RNA mislocalization starts, we analyzed ooplasmic streaming. This vigorous flow of oocyte cytoplasm from stage 10b to 12 is microtubule dependent and is inhibited by microtubule-depolymerizing drugs (Gutzeit, 1986; Theurkauf, 1994). However, we see normal ooplasmic streaming in *swa*, γ*Tub37C*, and *Dgrip75* mutants, strongly suggesting that the microtubules required for this process are not affected (see Supplemental Movies S4–S8 at http://www.developmentalcell.com/cgi/content/full/3/5/685/DC1). Therefore, this indicates that γ*Tub37C* and *Dgrip75* mutants affected only a subset of microtubules, which are required for *bcd* RNA localization, but not for ooplasmic streaming.

Evidence for an MTOC at the Anterior Pole at Stage 10b

One important question is whether there is a distinct MTOC responsible for localization of components at the anterior pole and, if so, what the molecular nature of such an MTOC might be. We used flies expressing the functional Dgrip75GFP fusion during oogenesis and found a substantial concentration of Dgrip75GFP at the anterior pole of wild-type oocytes. This concentration is first seen at stage 10b and becomes more pronounced during stage 11 (Figures 6A and 6B). Dgrip75GFP colocalizes with γ-tubulin, which was visualized with a pan anti-γ-tubulin antibody (Figures 6A and 6B). This anterior enrichment of γ-tubulin and Dgrip75GFP is not severely affected in *swa* mutant oocytes (Figure 6C). In contrast,

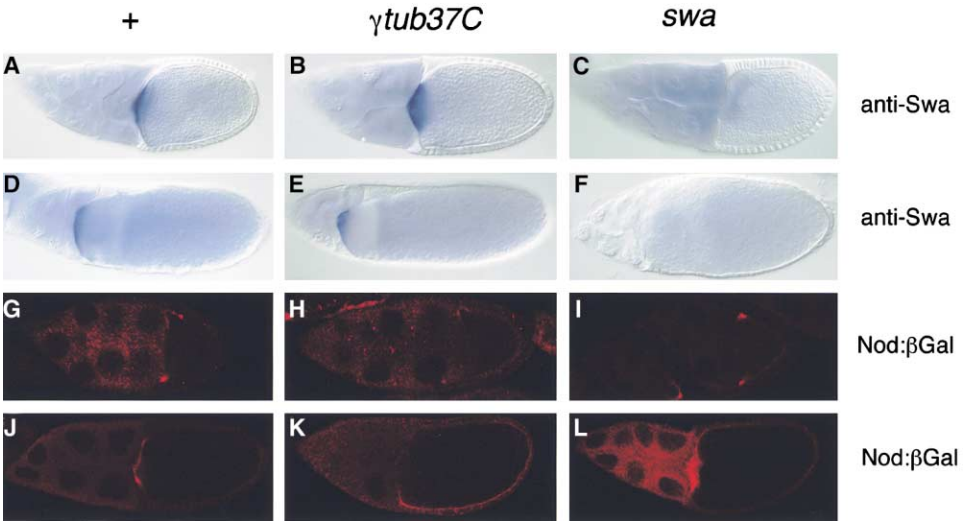

Figure 5. Analysis of Transport to the Anterior Pole

Swa (A–F) and Nod:β-gal staining (G–L) in wild-type egg chambers (A, D, G, and J) and in γ*Tub37C* (B, E, H, and K) and *swa* (C, F, I, and L) mutant egg chambers.

(A–C) Swa localization at stage 10b and at stage 12 (D–F) is normal in γ*Tub37C* mutants (B and E), whereas it is absent in swa^{VA11} mutants (C and F).

(G–I) Nod:β-gal is localized to the anterior corners of a wild-type and a *swa* mutant oocyte at stage 9 (G and I); in γ*Tub37C* mutants this localization is reduced (H).

(J–L) In wild-type (J) and *swa* mutants (L) Nod:β-gal concentrates at the entire anterior cortex at late stage 10b. This localization is not detectable in γ*Tub37C* mutant oocytes (K).

in γ*Tub37C* mutants, the γ-tubulin protein expression is severely reduced (Figure 6D), and, as a consequence, Dgrip75GFP is less concentrated at the anterior pole of a γ*Tub37C* mutant oocyte when compared with wild-type (Figures 6B and 6D). The anterior focus of γ-tubulin is neither due to overexpression of Dgrip75GFP nor established simply by dumping of material from nurse cells to the oocyte, since we detect a similar anterior enrichment of γ-tubulin in wild-type and *quail* mutants, which have a dumpless phenotype (Mahajan-Miklos and Cooley, 1994) (Figures 6E and 6G). Furthermore, we analyzed γ-tubulin in *gurken* (*grk*) mutants and found a distinct focus of γ-tubulin not only at the anterior pole, but also at the posterior pole, where it colocalizes with Swa. This suggests to us that γ-tubulin forms a distinct MTOC at both poles in *grk* mutants and only at the anterior pole in wild-type oocytes.

The analysis of α-tubulin in the oocyte at stage 10b and later is complicated by the fact that the microtubule-rich follicle cells surround the whole oocyte. Therefore, we expressed α-tubulin fused to GFP (Grieder et al., 2000), specifically in the germline, using maternal α-tubulin GAL4 and found microtubules enriched at the anterior pole at stage 10b and extending along the oocyte cortex (Figure 6I), as suggested before (Theurkauf et al., 1992). The anterior γ-tubulin focus overlaps with α-tubulin, consistent with the fact that γTub37C organizes microtubules from the anterior pole. In γ*Tub37C* and *Dgrip75* mutants, the anterior enrichment of α-tubulin appears reduced compared with wild-type; however, microtubules are still present in the mutants (Figure 6J–6L).

γTub37C Interacts with Swa

The colocalization of γTub37C and Dgrip75GFP at the anterior pole at stage 10b and their reported molecular interaction in cell culture (Fava et al., 1999) led us to analyze the interaction of γTub37C and Dgrip75 in ovary extracts. We are able to precipitate Dgrip75 from wild-type and *swa* mutant, but not from γ*Tub37C* and *Dgrip75* mutant ovaries, using the γTub37C-specific antibody (Figure 7A). Similarly, the Dgrip75 antibody precipitates γ-tubulin from wild-type and *swa*, but not from γ*Tub37C* and *Dgrip75* mutant ovaries (Figure 7B). This shows that γTub37C and Dgrip75 form a stable complex, presumably the γTuRC, during oogenesis. This complex is not disrupted in *swa* mutants. In addition to Dgrip75, Swa protein is also coimmunoprecipitated with the γTub37C antibody (Figure 7C). The binding of Swa to γTub37C is less stable than the γTub37C interaction with Dgrip75, suggesting that Swa is not a core component of the γTuRC. The Swa-γTub37C interaction is lost in *Dgrip75* and in all *swa* mutants analyzed. This shows that Dgrip75 is necesary for the efficient binding of Swa to γTuRC (Figure 7C). The mutant Swa proteins from swa^{TG31} or swa^{384} ovaries, which are not localized at the anterior pole of the oocyte (Schnorrer et al., 2000), do not bind to the γTuRC.

Discussion

swa Induces a Transition of the *bcd* RNA Localization Pattern

swa is essential for *bcd* RNA localization at mid-oogenesis, when large amounts of *bcd* RNA are imported from the nurse cells through ring canals into the oocyte (St Johnston et al., 1989; Stephenson et al., 1988). The *bcd* RNA localization pattern changes from a ring shape at stage 10a to a disc shape at stage 10b. This coincides with the colocalization of *bcd* RNA and Swa protein at the anterior cortex. Swa localization is

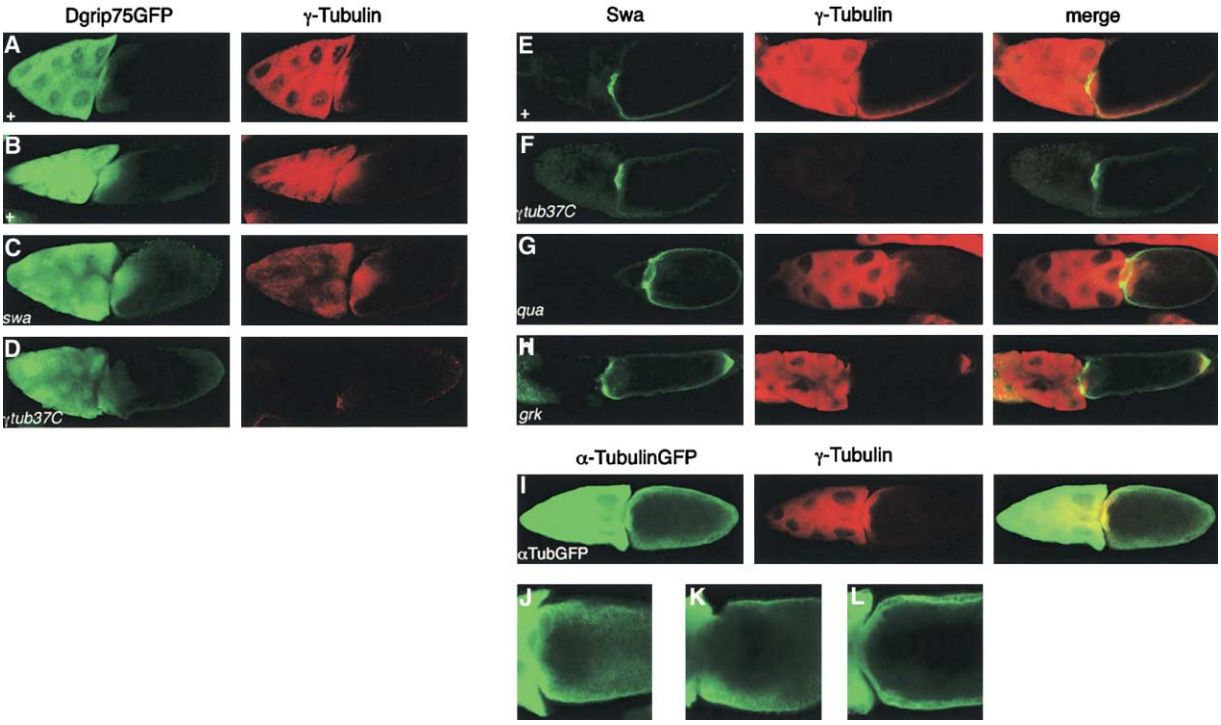

Figure 6. An Anterior MTOC at Midoogenesis

(A–D) Dgrip75GFP and γ-tubulin distribution during midoogenesis. Confocal images showing Dgrip75GFP in green with an anti-GFP antibody and γ-tubulin in red with a pan anti-γ-tubulin antibody in wild-type egg chambers (A and B) and in *swa* (C) and γ*Tub37C* mutant egg chambers (D). Dgrip75GFP and γ-tubulin are concentrated at the anterior cortex of wild-type oocyte at stage 10b (A). This concentration is increased at stage 11 (B) and not severely affected in a stage 11 *swa* oocyte (C). γ*Tub37C* mutants show strongly reduced Dgrip75GFP localization and γ-tubulin staining in a stage 11 oocyte (D).

(E–H) Swa and γ-tubulin distribution during midoogenesis. Confocal images showing Swa in green and γ-tubulin in red in wild-type egg chambers (E) and in γ*Tub37C* (F), *qua* (G), and *grk* (h) mutant egg chambers. Note the distinct posterior γ-tubulin focus in *grk* oocytes.

(I) α-tubulinGFP (green) and γ-tubulin (red) at stages 10b–11. Note the anterior overlap, whereas α-tubulin extends around the whole cortex.

(J–L) High-power analysis showing α-tubulinGFP in wild-type (J) and in γ*Tub37C* (K) and *Dgrip75* (L) mutants.

required for the transition of the *bcd* RNA localization pattern, since *bcd* RNA remains at the lateral cortex of stage 10b *swa* mutant oocytes and is never found in the middle at the anterior pole. *swa* likely has a specific function with respect to *bcd* RNA because, in *swa* mutants, the localization of other anteriorly concentrated components, like Nod:β-gal or the γTuRC components γTub37C and Dgrip75, is not as affected as *bcd* RNA. Thus, *swa* is not required for microtubule function in general. However, Swa might be involved in regulating the dynamics of certain microtubules, since the regular centrosome and spindle distribution during embryogenesis is affected in *swa* mutants.

Previously, we proposed that transport of Swa to the anterior pole of the oocyte occurs along microtubules via the minus end-directed dynein motor complex (Schnorrer et al., 2000). In support of this model, we find that SwaGFP localizes together with γTub37C and Cnn to centrosomes during early embryogenesis. At the end of mitosis the SwaGFP concentration at the centrosomes decreases and the localization is reestablished within the next interphase. This suggests that Swa is not an integral component of centrosomes, but localizes to microtubule minus ends in two different biological contexts. The distribution of the microtubule minus end directed motor Nod:β-gal at stage 10b of oogenesis suggests that transport to the microtubule minus ends persists at this stage. This supports the idea that also Swa achieves its localization by active transport. Nod:β-gal recapitulates the change in the localization pattern of *bcd* RNA. Therefore, this change is likely to be an active process, which, with respect to *bcd* RNA, requires localized Swa to function. But how is this change organized?

γ-Tubulin Ring Complex Is Essential for *bcd* RNA Localization

Our screening procedure for novel genes essential for *bcd* RNA localization identified mutations in γ*Tub37C* and *Dgrip75*, which affect *bcd* RNA localization at late stage 10b of oogenesis. These mutants are cytoskeletal factors that are essential for *bcd* RNA localization. They are both components of the same molecular complex, the γ-tubulin ring complex, and show the same *bcd* RNA mislocalization and early embryonic arrest phenotype. In the early embryo, the γ-tubulin ring complex is concentrated at the centrosomes, which organize the spindle microtubules. Other mutants in maternally expressed centrosomal proteins, which cause nuclear division problems during early embryogenesis, do not result in an aberrant *bcd* RNA distribution. We tested *centrosomin* (*cnn*) or *abnormal spindle* (*asp*) mutants (Gonzalez et al., 1990; Vaizel-Ohayon and Schejter, 1999) and detected no difference in *bcd* RNA localization

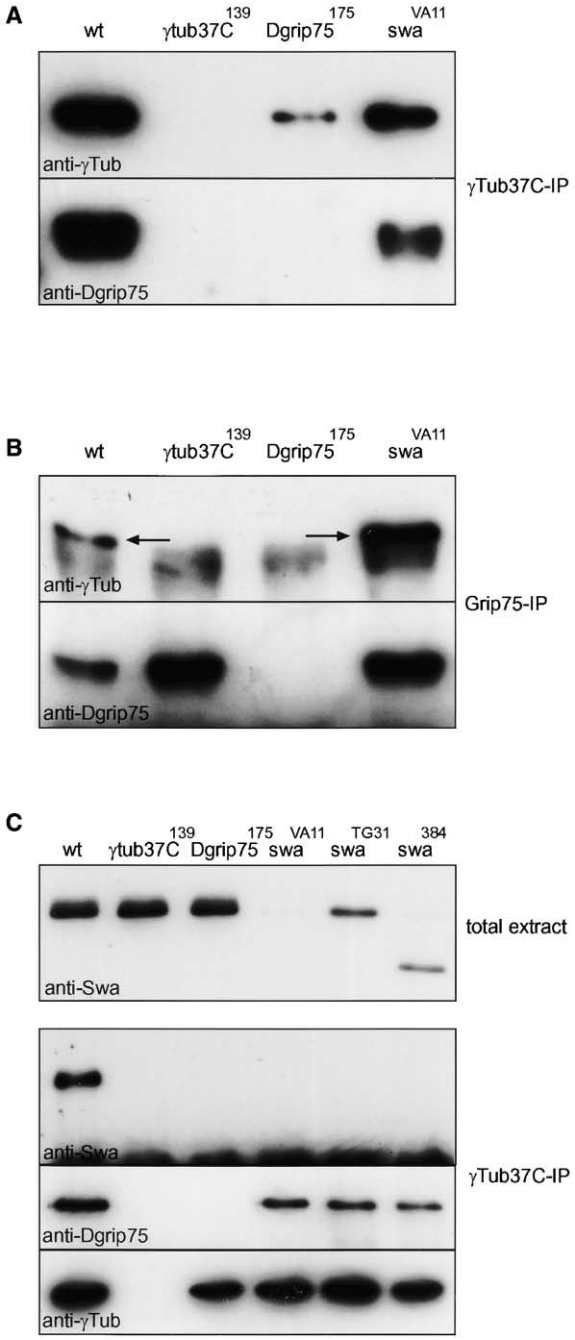

Figure 7. Coimmunoprecipitation of γTub37C, Dgrip75, and Swa

(A) The upper two panels show Western blots of an anti-γTub37C immunoprecipitation from wild-type ovary extracts and from γ*Tub37C*^{139}, *Dgrip75*^{175}, and *swa*^{VA11} mutant ovary extracts. The precipitate is probed with a mouse anti-γ-tubulin antibody (first panel) or the anti-Dgrip75 antibody (second panel). Dgrip75 is coimmunoprecipitated with γTub37C from wild-type and *swa* mutant extracts, but not from γ*Tub37C* or *Dgrip75* mutant extracts.

(B) Immunoprecipitation as in (A), but with the anti-Dgrip75 antibody. γ-tubulin is coimmunoprecipitated with Dgrip75 from wild-type and *swa* mutant extracts, but not from γ*Tub37C* or *Dgrip75* mutant extracts.

(C) Immunoprecipitation as in (A) with anti-γTub37C antibody from wild-type and different mutant extracts. The upper panel shows the Swa protein load in the total extract. The lower two panels show that Swa is coimmunoprecipitated with γTub37C only from wild-type extract, but not from *Dgrip75* or *swa* mutant extracts.

(data not shown). This demonstrates that our screening procedure is stringent enough to identify specific factors, and, since it covered only the left arm of the second chromosome, it is likely that more genes with a function in *bcd* RNA localization exist in the genome.

γ*Tub37C* and *Dgrip75* Have Specific Functions in Microtubule Organization

γ*Tub37C* and *Dgrip75* mutants are required for microtubule assembly during embryogenesis and, therefore, are necessary for starting the nuclear divisions (Llamazares et al., 1999; Tavosanis et al., 1997). However, both mutants are viable and, hence, not essential for the microtubule-dependent processes during later embryonic and larval development. For γ*Tub37C* this is less surprising because it is only expressed maternally and a second γ-tubulin, γ*Tub23C*, fulfils zygotic functions (Sunkel et al., 1995). In contrast, *Drosophila* has no second paralog of *Dgrip75* in the genome. Consequently, *Dgrip75* plays an essential role only in certain microtubule-dependent processes, which appear to be those that also require γ*Tub37C*. However, both *Dgrip75* alleles are sterile not only in females, but also in males, a phenotype described for mutants in *centrosomin*, but not in γ*Tub37C* or *swa* (Li et al., 1998, our unpublished data; Tavosanis et al., 1997).

γ*Tub37C* and *Dgrip75* mutants display a specific loss of microtubule function during oogenesis. Several microtubule-dependent processes, such as oocyte specification, nuclear migration, and *osk* and *bcd* RNA transport at stage 9, are functional. *bcd* RNA mislocalization starts at late stage 10b. This phenotype can be explained in two ways. Either γ*Tub37C* and *Dgrip75* affect all microtubules of the oocyte at a specific time point or they eliminate the function of only a subset of microtubules, while others are unaffected. The latter explanation, which proposes specialized microtubules, is supported by the fact that a maternal α-*tubulin* exists that has a relatively divergent primary sequence and comprises about 20% of the α-tubulin pool in oocytes (Matthews et al., 1993; Theurkauf, 1992). Furthermore, tubulin modifications such as acetylation or polyglycylation may distinguish certain microtubules from others (for review see Rosenbaum, 2000). We tried to address this problem by analyzing microtubule-dependent transport to the posterior pole. Posterior transport is normal in γ*Tub37C* mutants; however, it is possible that this transport is already completed at stage 10b, since Kin:β-gal is no longer concentrated at the posterior at late stage 10b (data not shown; Clark et al., 1994). Hence, posterior transport might not take place anymore and does not allow us to distinguish between the two possibilities proposed above. Therefore, we analyzed ooplasmic streaming, a microtubule-dependent process that occurs at the same time as the *bcd* RNA mislocalization in γ*Tub37C*, *Dgrip75*, and *swa* mutants. The fact that ooplasmic streaming is unaffected in all the mutants suggests that only a subset of microtubules,

which are required for *bcd* RNA localization from stage 10b onward, but not for ooplasmic streaming, are affected. This conclusion is directly supported by the α-tubulin analysis in γ*Tub37C* and *Dgrip75* mutants, which shows the presence of microtubules in these mutant oocytes at stage 10b.

Evidence for an Anterior MTOC at Stage 10b Essential for *bcd* RNA Localization

So far, no microtubule-organizing center (MTOC) has been described at the anterior pole at any stage of oogenesis that would allow directed microtubule-dependent transport to the anterior pole and demonstrate the polarity of the microtubule network. The γ-tubulin ring complex is capable of nucleating microtubules in a controlled manner and, in addition, can act as a cap that stabilizes their minus ends (Moritz et al., 1995; Wiese and Zheng, 2000; Zheng et al., 1995). γ*Tub37C* and *Dgrip75* are essential for these functions of the ring complex during embryogenesis and, most likely, also during oogenesis. Since Dgrip75GFP is a functional component of the γTuRC in the embryo and stably binds to γTub37C during oogenesis, the colocalization of Dgrip75GFP and γTub37C at the anterior pole of a stage 10b wild-type oocyte suggests that Dgrip75 and γTub37C form a distinct microtubule organizer at the anterior pole at stage 10b. Importantly, *grk* mutants show an ectopic posterior γTub37C focus, consistent with the model of microtubule minus ends at both poles in *grk* oocytes. Furthermore, this posterior γTub37C focus supports the idea that the γTub37C focus at the anterior pole in wild-type oocytes is not simply a consequence of dumping the nurse cell cytoplasm into the oocyte, but a distinct MTOC. This is further supported by the anterior enrichment of γTub37C in dumpless mutants. The proposed anterior MTOC might have a similar molecular composition as the γTuRC during embryogenesis and interacts with Swa protein. The disrupted *bcd* RNA and Nod:β-gal localization in *Dgrip75* and γ*Tub37C* mutants shows the functional significance of the proposed MTOC.

The formation of a γ-tubulin ring complex-based MTOC in the middle of the anterior pole at stage 10b may explain the "ring to disc" transition of the localization pattern of *bcd* RNA and Nod:β-gal and is consistent with the anterior colocalization of γTub37C and α-tubulin. However, α-tubulin is not restricted to the anterior cortex at stages 10b–11 but extends along the whole oocyte cortex (see Figure 6I and Theurkauf et al., 1992). It is likely that the γ-TuRC functions as a template to nucleate certain microtubules at the anterior pole in a controlled direction.

Swa and *bcd* RNA Localization Are Uncoupled

An interesting aspect of the γ*Tub37C* and *Dgrip75* mutant phenotypes is that they uncouple Swa and *bcd* RNA localization. Since the defect in *bcd* RNA localization starts slightly later in γ*Tub37C* and *Dgrip75* than in *swa* mutants, we expected Swa to be localized, which is the case. But the colocalization of Swa and *bcd* RNA is lost at late stage 10b, arguing against the hypothesis that Swa binds directly to *bcd* RNA in order to transport it to, or anchor it at, the anterior pole. Furthermore, it is unlikely that Swa itself acts as molecular anchor to trap *bcd* RNA, which is imported from the nurse cells. The situation at the anterior pole appears to be more complicated, and we propose the following model.

In a first phase lasting until stage 10a, *bcd* RNA is localized in an *exu*- and microtubule-dependent process to the anterior pole into a ring-shaped pattern. This requires neither γ*Tub37C* and *Dgrip75* nor *swa* to function. In a second phase, a change in microtubule organization occurs, microtubules are mainly assembled subcortically, and transport to the posterior pole stops. Swa protein localization starts at the entire anterior cortex of the oocyte, not only in its corners, possibly in a dynein-dependent manner. The *bcd* RNA localization pattern changes into a disc or a cap-like pattern at the anterior pole. This is likely to be an active process, which initially does not depend on γ*Tub37C* and *Dgrip75* but does depend on *swa*. Swa might use γTub23C and other Grips in order to partially reorganize the microtubule cytoskeleton. In a third phase, starting at late stage 10b, γ*Tub37C* and *Dgrip75* are essential to keep *bcd* RNA anteriorly and to complete the ring- to disc-shape transition. During this time the localized amount of *bcd* RNA increases continuously. Since Dgrip75GFP and γTub37C are enriched at the anterior pole from stage 10b onward, we propose that an MTOC, which might organize a subset of microtubules with distinct polarity, is established there at this time. In γ*Tub37C* and *Dgrip75* mutants, this MTOC is disrupted and *bcd* RNA diffuses into the oocyte. This is presumably promoted by large amounts of nurse cell cytoplasm entering the oocyte through the ring canals at the anterior pole. Therefore, *bcd* RNA either requires a stable anchor or continuous transport back to the anterior pole during phases two and three. We favor the hypothesis of continuous transport along microtubules, considering the molecular nature of γ*Tub37C* and *Dgrip75*.

In contrast to *bcd* RNA, Swa stays localized at the anterior cortex in γ*Tub37C* and *Dgrip75* mutants. This argues that Swa itself is less sensitive to microtubule disruption than *bcd* RNA and might be anchored at the anterior cortex after its initial localization. The localized amount of Swa does not increase anymore after stage 10b. Swa binds to the γ-TuRC and might regulate microtubule turnover or dynein motor recycling. This binding is lost in *swa* mutants, demonstrating that the C terminus of Swa is required for the interaction with the γ-TuRC. The possibility that Swa itself has the capacity to change properties or location of a MTOC remains to be tested. In the future, we should be able to integrate proteins like Exu and Swa, which seem to have specialized functions for only a few processes and are poorly conserved during evolution, into the cellular machinery required for asymmetric localization of determinants. This machinery requires a variety of conserved cytoskeletal components, which can be used for mRNA transport at different developmental stages, such as *Drosophila* oogenesis and embryogenesis (Bullock and Ish-Horowicz, 2001).

Experimental Procedures

Fly Strains

The wild-type stock was Oregon R. The following mutant or transgenic strains were used: swa^{99}, swa^{VA11}, swa^{TG31}, swa^{384} (Schnorrer

et al., 2000), and *swa* deficiency *Df(X) JF-5* (Stephenson et al., 1988) (*swa* mutants were either homozygous or transheterozygous with *Df(X) JF-5*; *γTub37C*^{139}; *Dgrip75*^{175} and *Dgrip75*^{224} were induced as described below); *γTub37C*^{1}, *γTub37C*^{3}, and *γTub37C* deficiencies *Df(2L)TW150*, *Df(2L)TW158*, *Df(2L)TW130*, and *Df(2L)VA16*; *Dgrip75* deficiencies *Df(2L)J39*, *Df(2L)J-der27*, and *Df(2L)J-der2* (Bloomington); *cnn*^{HK21} (Vaizel-Ohayon and Schejter, 1999); *asp*^{dd1} and *asp*^{dd3} (White-Cooper et al., 1996); *grk*^{DC}, *grk*^{HK}, *qua*^{WP}, and *qua* deficiency *Df(2L)TW137* (Tübingen stock collection); KZ503 and NZ143.2 (Clark et al., 1997); α-TubGFP (Grieder et al., 2000); FRT40A(2L) (Xu and Rubin, 1993); hs-Flp122(X) (Struhl and Basler, 1993).

Screening Procedure

The screening procedure will be described in detail in S.L. and C.N.-V. (in preparation). In brief, males carrying a marked al dp b pr FRT40A(2L) chromosome were mutagenized with ethyl methane sulfonate (EMS) and crossed to females homozygous for hs-Flp122 (X) and an FRT40A chromosome carrying two P[ubi-nlsGFP, w^{+}] insertions on the left arm. Larvae from this cross were heat-shocked to induce germline clones and raised to adulthood. From single F_1 females the nongreen embryos (homozygous clones for the mutagenized arm 2L) were screened for cuticle structures (Luschnig et al., 2000). In a secondary screen, we analyzed a class of mutants that showed early arrest phenotypes, as indicated by a lack of cuticle structures. If the female produced three or more clonal eggs, which did not develop, a line was established. We used the Flp-ovo^{D} system to induce germline clones (Chou and Perrimon, 1996) and screened ovaries by in situ hybridization with *bcd* and *osk* RNA antisense probes. From a total of 6760 females with mutations on chromosome arm 2L, 49 lines were isolated that showed no cuticle formation after retesting the lines.

Identification of *Dgrip75* Mutants and Rescue with Dgrip75GFP Fusion

The female sterility of *175-14* and *224-26* was mapped to 31D-F with chromosomal deficiencies *Df(2L)J39*, *Df(2L)J-der27*, and *Df(2L)J-der2*. The genomic DNA of the *Dgrip75* gene from start to stop codon was amplified from *175-14*, *224-26*, and the unmutagenized parental chromosome with primers 5′-TCAGGATCCCAATGATA CACGATTTGTTATTGG-3′ and 5′-TCAGCGGCCGCTCATGCGGAC GTATTATGACTG-3′ with Taq Polymerase (Amersham-Pharmacia) and sequenced by standard techniques. Sequence alignment was performed with Lasergene software (DNA-Star). For the rescue construct, *Dgrip75* was amplified from genomic DNA starting with the 5′-UTR to the last amino acid with primers 5′-TCAGCGGCCGCCG CAAATGAAATACAAATACCG-3′ and 5′-TCATCTAGAGCTAGCTGC GGACGTATTATGACTGG-3′, cut with NotI, NheI, and fused to GFP, which was amplified with primers 5′-TCAGCTAGCATGAGTAAAG GAGAAGAACTT-3′ and 5′-TCATCTAGATTAGTATAGTTCATCCAT GCC-3′ and cut with NheI, XbaI. The fusion was cloned into the transformation vector pUASp (Rorth, 1998) with NotI, XbaI, and transgenic flies were created by standard techniques. The insertion Dgrip75GFP-3 (3rd chromosome) was recombined on an α-*tubulin*-driven Gal4 chromosome (line V15, Daniel St Johnston) and crossed into *Dgrip75*^{175} background.

Generation of Dgrip75 Antisera and Western Analysis

Two internal fragments of *Dgrip75* (amino acids 58–115 and 175–406) were cloned into pQE-30 (Qiagen). The fusion proteins were overexpressed in *E. coli* and purified on nickel agarose columns (Qiagen). The two purified fragments were mixed, suspended in 1 vol Freund's adjuvant (Sigma) and injected into rabbits. After the initial immunization, rabbits were boosted and bled in 2 week intervals. The antisera were partially purified against His-tagged fusion protein immobilized on CNBr-activated Sepharose (Amersham-Pharmacia). For Western analysis hand-dissected ovaries were boiled in 20 μl Lämmli buffer per milligram of ovaries. Five to 10 μl extract was loaded on a minigel, blotted to a PVDF membrane (Millipore), blocked with milk powder, and incubated with the primary antibody at 4°C overnight (anti-Swa serum 1:20000; anti-Dgrip75 affinity purified 1:2000; anti γTubC12 1:5000 (Moritz et al., 2000); mouse anti-γTub 1: 5000 [Sigma]). The blot was developed with a peroxidase-conjugated secondary antibody (Dianova) and the ECL system (Amersham-Pharmacia).

Immunoprecipitations

Ovaries were hand dissected, homgenized in 10 μl IPP150 (20 mM Hepes [pH 8.0] and 150mM NaCl) plus 0.5% NP40 per microgram of ovaries, extracted with $C_2F_3Cl_3$, and centrifuged for 15 min. Fifty microliters of ovary extract was incubated with 10 μl protein A Sepharose (Pharmacia) and 0.3 μl antibody for 3 hr at 4°C. Beads were washed four times with IPP150 plus 0.1% NP40 and boiled in 25 μl Lämmli buffer. Ten microliters were used per lane for Western analysis.

In Situ Hybridization and Antibody Stainings

For in situ hybridizations ovaries were fixed in 4% paraformaldehyde (PFA) in PBS plus 0.1% Tween 20 (PBT)/Heptane for 20 min, transferred to methanol, and stored at −20°C overnight. After rehydration and refixation with 4% PFA, ovaries were digested with 50 μg/ml proteinase K (Merck) for 10 min, incubated in 2 mg/ml glycine for 2 min, washed four times with PBT, and refixed in 4% PFA. After being washed with PBT and transfered into hybridization solution (hyb) (50% formamide, 5 × SSC, 0.2% Tween 20), ovaries were blocked for 1 hr in hyb plus torula RNA plus 50 μg/ml heparin at 68°C and incubated with DIG-labeled *bcd* or *osk* RNA antisense probes at 68°C overnight. Ovaries were washed twice for 30 min with hyb and for 15 min each with hyb:PBT 3:2 and 2:3 at 68°C, blocked with 5% normal goat serum (Dianova), incubated with alkaline phosphatase-conjugated anti-DIG antibody (1:5000; Dianova) for 2 hr, and stained with NBT/X-Phos. For antibody stainings ovaries were dissected, fixed for 20 min in 4% PFA in PEMS (0.1 M PIPES [pH 6.9], 2 mM $MgSO_4$, and 1 mM EGTA [pH 8.0])/heptane (with the exception of Figures 6E–6L, in which heptane was omitted), washed in PBT, transferred into methanol, and rehydrated into PBT. Proteinase K digestion was done as described above, and then ovaries were blocked for 1 hr in 5% normal goat serum and incubated with the primary antibody at 4°C overnight (anti-Swa affinity pure 1:1000 and anti-β-gal 1:2000 [Promega], anti-GFP 1:1000 to 1:5000 [Torry Pines Biolabs], mouse anti-γTub 1:5000 [Sigma]). Ovaries were incubated with secondary antibodies obtained from Dianova (anti-mouse or -rabbit alkaline phosphatase, Cy3 or Cy5 conjugated) or Molecular Probes (Alexa 488-conjugated anti-mouse or -rabbit) for 2 hr at room temperature. For visualization of fluorescent stainings, a Leica TS confocal microscope was used.

Time-Lapse Imaging

For GFP time-lapse movies, embryos were dechorionated in 50% Clorix (bleach), washed, and embedded in 3S Voltalef oil (Atochem). Frames were taken every 15 s with a Leica TS confocal microscope. For ooplasmic streaming, ovaries were dissected and embedded in 10S Voltalef oil (Atochem). Frames were taken every 10 s with a Zeiss Axioplan 2 and a Hamamatsu digital camera. The movies were assembled with Metamorph software.

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